1083-8791/05/1112-0006$30.00/0
doi:10.1016/j.bbmt.2005.08.038

A CD4 Domain 1 CC′ Loop Peptide Analogue Enhances Engraftment in a Murine Model of Bone Marrow Transplantation with Sublethal Conditioning

Gabor Varadi,[1] *Thea M. Friedman,*[2] *Robert Korngold*[2]

[1]Kimmel Cancer Center, Jefferson Medical College, Philadelphia, Pennsylvania; [2]Cancer Center, Hackensack University Medical Center, Hackensack, New Jersey

Correspondence and reprint requests: Robert Korngold, PhD, Cancer Center, Hackensack University Medical Center, Jurist Research Building, 30 Prospect Ave., Hackensack, NJ 07601 (e-mail: rkorngold@humed.com).

Received July 5, 2005; accepted August 22, 2005

ABSTRACT

Host CD4$^+$ T cells that survive sublethal or even lethal preconditioning regimens can participate in the process of hematopoietic stem cell graft rejection, particularly when the transplantations are performed across a major histocompatibility complex (MHC) class II barrier. To enhance donor marrow engraftment, we tested the efficacy of a small synthetic cyclic heptapeptide, 802-2 (CNSNQIC), which was designed to closely mimic the CD4 domain 1 CC′ surface loop, theoretically involved in CD4/MHC class II complex oligomerization and subsequent CD4$^+$ T-cell activation. Previously, this peptide was found to have inhibitory activity in murine models for CD4$^+$ T cell–dependent graft-versus-host disease and skin allograft rejection. Herein, we used the MHC class II–disparate bm12 → B6-CD45.1 sublethal irradiation transplantation model to test the possibility that the 802-2 peptide could enhance the engraftment of donor T cell–depleted bone marrow (ATBM). Sublethally irradiated B6-CD45.1 mice that received bm12 ATBM in combination with the 802-2 peptide demonstrated increased donor marrow cell engraftment as compared with mice that received ATBM alone; this suggests that the 802-2 peptide may be useful as an immunomodulating agent to overcome MHC class II mismatch barriers in hematopoietic stem cell transplantation.

KEY WORDS

CD4 • Peptide analogue • Hematopoietic stem cell transplantation • Engraftment

INTRODUCTION

Allogeneic blood and bone marrow transplantation (BMT) can be a potentially curative treatment for several malignant and nonmalignant hematologic diseases, although several major obstacles may hamper an overall successful outcome. These complications include graft failure, opportunistic infections, relapse of malignancy, and either acute or chronic graft-versus-host disease (GVHD). In the case of GVHD, mature alloreactive donor T cells in the graft can mediate considerable tissue damage and lead to significant morbidity and mortality [1,2]. Conversely, donor T cells are important for ensuring engraftment by targeting residual host elements, can mount antitumor effects, and can stave off opportunistic infections after transplantation [3]. To reconcile these conflicting roles of the donor T cells, BMT strategies must be developed in an effort to retain the positive capabilities while reducing or controlling the deleterious effects. Graft failure remains an important part of the equation in consideration for improved BMT outcomes, as evidenced in the recent development of nonmyeloablative conditioning regimens to reduce the incidence of transplant-related mortality and to promote hematopoietic chimerism. In this case, although the approach holds great promise for making the transplantation option available to a much broader range of patients, it has unfortunately also led to a significantly higher risk of graft rejection [4-6].

The elucidation of the detailed interactive nature of antigenic peptide/major histocompatibility complex (MHC) molecules on antigen-presenting cells with T-cell receptor/CD4 complexes on helper CD4$^+$ T cells and of additional co-receptors has established a

foundation for developing novel approaches to mediate graft acceptance [7]. Modalities for achieving allograft tolerance have included blocking the ligation of CD40/CD40 ligand molecules on the surface of dendritic antigen-presenting cells and blocking the ligation of CD28/B7-1 and B7-2 molecules on T cells, either directly by use of antibodies or by use of soluble cytotoxic T lymphocyte antigen 4 (CTLA-4) molecules [8,9]. Other promising therapeutic targets aim at disrupting the putative antigen-induced oligomerization process among CD4 molecules, thus rendering the T cells anergic [10].

We have previously used a small synthetic cyclic heptapeptide, 802-2 (CNSNQIC), which was designed to closely mimic the CD4 domain 1 CC′ surface loop, theoretically involved in CD4/MHC class II complex oligomerization and subsequent $CD4^+$ T-cell activation [11]. This peptide was found to have an inhibitory effect in MHC-mismatched murine models on the development of $CD4^+$ T cell–dependent GVHD and skin allograft rejection [11] and could inhibit the development of $CD4^+$ T cell–mediated autoimmunity in models for experimental allergic encephalomyelitis [12]. Herein we used the MHC class II–disparate bm12 → B6-CD45.1 sublethal irradiation BMT model to test the possibility that the 802-2 peptide could enhance the engraftment of T cell–depleted bone marrow (ATBM) by preventing the residual host $CD4^+$ T cell–mediated rejection of donor hematopoietic stem cells. Recipient mice were exposed to 800 cGy of irradiation and administered phosphate-buffered saline (PBS), a single intravenous dose of 802-2 peptide, or anti-CD4 monoclonal antibody (mAb) at the time of BMT. Donor-host chimerism was assessed 1 to 2 months after transplantation by flow cytometric analysis of spleen and/or lymph node cells. Both 802-2 peptide–treated and anti-CD4 mAb–treated recipients exhibited enhanced donor lymphoid engraftment, with 70% to 80% donor chimerism, as compared with 20% to 30% for the PBS-treated control mice. Furthermore, engraftment of donor hematopoietic progenitor cells in the spleens of recipients was assessed by a 6-day colony-forming unit–granulocyte macrophage (CFU-GM) assay, in which 802-2 peptide–treated animals yielded enhanced numbers of donor colonies. Taken together, these results suggest that the 802-2 peptide may be useful as an immunomodulating agent to overcome MHC class II mismatch barriers in BMT.

MATERIALS AND METHODS

Mice

MHC class II mutant B6.C-$H2^{bm12}$ (bm12) mice (which express the CD45.2 marker) were purchased from the Jackson Laboratory (Bar Harbor, ME), and B6-Ly5.2 mice (which actually express the CD45.1 marker and are referred to herein as B6-CD45.1 mice to avoid confusion in the nomenclature) were purchased from the National Cancer Institute (Bethesda, MD). Male bm12 mice, 7 to 12 weeks of age, were used as bone marrow donors, and male B6-CD45.1 mice, 7 to 10 weeks of age, were used as recipients. Mice were kept in a sterile environment in microisolator cages at all times and were provided with acidified water and autoclaved food.

Media

PBS solution supplemented with 0.1% bovine serum albumin (BSA; Sigma Chemical, St. Louis, MO) was used for all in vitro manipulations of the donor bone marrow cells and lymphocytes. For intravenous injection, all cells were resuspended in PBS. RPMI 1640 medium (Mediatech, Herndon, VA) was used for all in vitro assays, supplemented with 10% fetal calf serum (Atlanta Biologicals, Norcross, GA), 2 mmol/L glutamine, 50 U/mL penicillin, 50 mg/mL streptomycin, and 0.05 mmol/L 2-mercaptoethanol (Mediatech). Methocult M3230 (Stemcell Technologies, Vancouver, BC, Canada) methylcellulose medium was used for all in vitro colony assays, supplemented with 50 U/mL interleukin 3 (Biosource International, Camarillo, CA), 2 mmol/L glutamine, 50 U/mL penicillin, and 50 mg/mL streptomycin (Mediatech).

802-2 Peptide

The cyclized 802-2 peptide (CNSNQIC), designed as previously described [11], was synthesized on an Applied Biosystems 430A peptide synthesizer (Foster City, CA) and a model 9050 Pepsynthesizer Plus (Perspective Biosystems, Cambridge, MA) by solid-phase synthesis with a standard F-moc strategy. The purity of the final product was assessed by analytical reverse-phase and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. The peptide was reconstituted in PBS (2.5 mg/mL) for injection into mice.

Irradiation

All recipient mice received appropriate sublethal total body irradiation exposures of 650 to 900 cGy by using a Mark-I-68A cesium 137 source (143 cGy/min; JL Shepherd, San Francisco, CA).

Monoclonal Antibodies

Ascites fluid for anti-Thy1.2 (J1j.10, rat immunoglobulin [Ig]M; American Type Culture Collection [Manassas, VA] TIB184) [13] and anti-CD4 (GK1.5, rat IgG2b; American Type Culture Collection TIB-207) [14] mAbs was generated in our laboratory from intraperitoneal (IP) injection of the hybridomas into BALB/c nu/nu mice (National Cancer Institute). Goat

anti-mouse IgG antibody was purchased from Cappel-Organon Teknika (Durham, NC), and FcR blocking mAb [15]was purchased from Pharmingen (San Diego, CA). Surface phenotype was analyzed by dual-color immunofluorescence by using the following fluorescein isothiocyanate (FITC)–conjugated and/or R-phycoerythrin (PE)–conjugated anti-mouse mAbs, purchased from Pharmingen: rat anti-CD45.1 (clone A20-1.7), rat anti-Thy1.2 (CD90; clone 30-H12), rat anti-CD4 (clone RM4-5), rat anti-CD8a (clone 53-6.7), rat anti-CD45R/B220 (clone RA3-6B2), mouse anti-CD45.2 mAb (clone 104), and, as a negative control, rat IgG2a*k*.

Cell Preparations

Bone marrow cells were prepared from the femurs and tibiae of donor mice by flushing with PBS/BSA. To prepare anti-Thy1.2 mAb–treated (T cell–depleted) bone marrow (ATBM), cells were incubated with J1j.10 mAb (1:100 dilution) and guinea pig complement (1:20) for 45 minutes at 37°C and were washed 3 times. ATBM cells were counted and resuspended at 5×10^7/mL in PBS. This treatment resulted in a donor cell population deficient of Thy1.2$^+$ cells, as determined by immunofluorescence/flow cytometry.

Bone Marrow Engraftment

In an adaptation of the bone marrow engraftment model used by Vallera et al. [16], recipient mice were exposed to an appropriate level of sublethal total body irradiation 4 to 6 hours before injection of donor ATBM cells. Mice were then given a 0.2-mL injection of PBS (IV), 802-2 peptide (0.5 mg IV), or anti-CD4 mAb (1:100 dilution of GK1.5 ascites fluid, 25 μg, IP, ×2). Chimerism was analyzed at 1 and 2 months after transplantation. For presensitization experiments, recipient B6-CD45.1 mice were injected IP with 2×10^7 bm12 splenocytes 14 days before irradiation and BMT.

Chimerism Phenotyping

FITC–anti-CD45.1 and PE–anti-CD45.2 mAb, along with T- or B-cell differentiation antigens, were used to evaluate the phenotype and the degree of chimerism in the spleens (at 1 month) or in the spleens and lymph nodes (at 2 months) of bm12 → B6-CD45.1 recipients. For analysis, single-cell suspensions (1×10^6 cells per sample) were incubated and washed with PBS/0.1% BSA and 0.05% NaN_3 (fluorescence-activated cell-sorter buffer). Cell samples in a volume of 100 μL were initially incubated with 25 μL of anti-Fcγ receptor mAb for 10 minutes at 4°C to prevent nonspecific Fc binding before further incubation with phenotypic mAb [15]. Samples were analyzed on an XL-MCL flow cytometer (Beckman Coulter, Miami, FL): a minimum of 10^4 cells was analyzed for each determination.

Assay for Hematopoietic Progenitors and Phenotypic Analysis

The CFU-GM assay was performed according to a modification of a method described previously [17,18]. Briefly, splenocytes were collected and pooled from 3 recipient mice per treatment group on day 5 after transplantation, 1×10^5 cells per group were plated in triplicate 35-mm culture dishes in 1 mL of Methocult media, and cultures were maintained at 37°C in 7% carbon dioxide for 6 days. Cell aggregates that contained >30 cells (CFU-GM) were scored as individual colonies on day 6 of culture, and after a further 2 to 4 days of culture, 6 to 9×10^2 cell aggregates were extracted from the methylcellulose and pooled from each treatment group, yielding approximately 2×10^5 cells. These cells were then phenotyped by dual-color flow cytometry with PE–anti-CD45.1 and FITC–anti-CD45.2 mAb to determine the origin of the CFU-GM cells.

Statistical Analyses

The mean percentage expressions of CD45.2 (donor) or CD45.1 (recipient) on cells in the experimental populations were compared with each other for significance by the unpaired *t* test.

RESULTS

Radiation Dose Titration Effect on Donor Engraftment

Dose titration experiments were performed to determine the optimal level of preconditioning radiation exposure of B6-CD45.1 recipients to ensure maximum residual host resistance to the engraftment of 1×10^7 donor bm12 ATBM cells. Flow cytometric analysis of splenocytes was used to measure the percentage of donor chimerism at 30 days after BMT. Mice that weighed 25 to 29 g and were exposed to sublethal 750 to 800 cGy of radiation exhibited approximately 20% donor chimerism (Figure 1). Recipient mice that received radiation doses ≥850 cGy exhibited a significant increase (65%; $P < .014$) in donor chimerism. It was also found that the weight of the mice played an important role in determining the optimal radiation dose. For mice that weighed 22 to 25 g, similar donor cell engraftment levels (approximately 65%) were attainable at an exposure of 800 cGy (data not shown). Therefore, proper irradiation doses were chosen and adjusted for the weights of the recipient mice in each experiment.

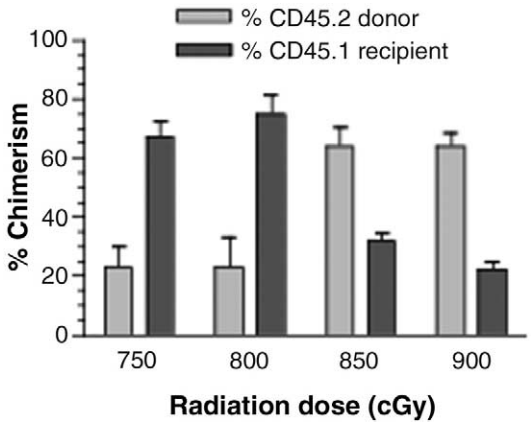

Figure 1. Radiation dose titration effect on donor engraftment in the MHC class II–mismatched bm12 → B6-CD45.1 strain combination. B6-CD45.1 mice (n = 3-5) were exposed to irradiation with the indicated dosage and underwent transplantation with allogeneic bm12 ATBM (10^7 cells). Splenocytes from recipients were analyzed for donor chimerism on day 30 after BMT by flow cytometry with FITC–anti-CD45.1 (expressed by recipient) and PE–anti-CD45.2 (expressed by donor) mAb. The data are presented as the mean percentage of chimerism ± SD and are from 1 representative experiment of 2 performed.

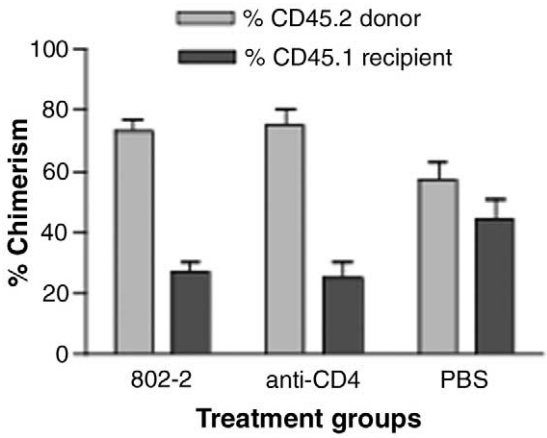

Figure 3. Splenic donor mononuclear cell engraftment 72 days after BMT. B6-CD45.1 mice underwent transplantation with bm12 ATBM and were treated as described in Figure 2. Mononuclear cells from recipient spleens (n = 3-5) were analyzed for donor chimerism on day 72 after BMT. The donor expressions in the 802-2 peptide (73.5%) and anti-CD4 mAb (75.0%) treatment groups were significantly higher than in the PBS control group (38.3%; $P < .03$). The data (mean ± SD) represent the mean of 2 experiments.

Effect of 802-2 Peptide on Donor Mononuclear Cell Engraftment

To investigate the effect of the 802-2 peptide on enhancement of donor bone marrow engraftment, sublethally irradiated (800 cGy) B6-CD45.1 mice transplanted with 10^7 bm12 ATBM cells received an injection of PBS (0.2 mL IV), 802-2 peptide (0.5 mg; 0.2 mL; IV), or anti-CD4 mAb (25 μg; 0.2 mL; IP). Flow cytometric analysis 30 days after BMT indicated 38.3% donor engraftment of donor origin $CD45.2^+$ mononuclear cells in the spleens of the PBS control group, whereas both the 802-2 peptide and anti-CD4 mAb treatment groups had an approximately 2-fold increase of donor cell engraftment (76.5% and 80.1%, respectively; $P < .001$; Figure 2).

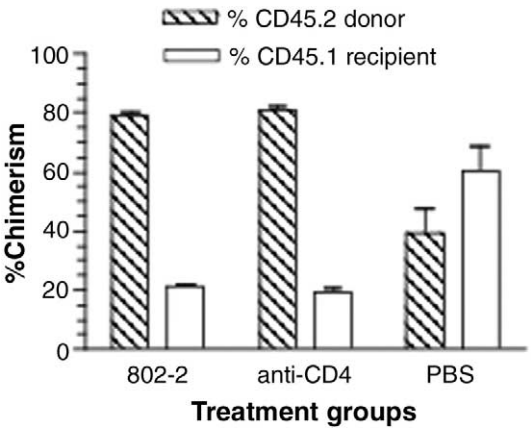

Figure 2. Effect of 802-2 peptide on splenic donor mononuclear cell engraftment 30 days after BMT. B6-CD45.1 mice (n = 3-5) were exposed to 800 cGy of irradiation and underwent transplantation with allogeneic bm12 ATBM (10^7 cells). Recipients were treated on day 0 with PBS, 802-2 peptide (0.5 mg), or anti-CD4 mAb. Mononuclear cells from recipient spleens were analyzed for donor/recipient chimerism on day 30 after BMT by flow cytometry with FITC–anti-CD45.1 and PE–anti-CD45.2 mAb. The donor expressions in the 802-2 peptide (76.5%) and anti-CD4 mAb (80.1%) treatment groups were significantly higher than in the PBS control group (38.3%; $P < .001$). The data (mean ± SD) are from 1 representative experiment of 2 performed.

At 72 days after BMT, 57.2% of the splenocytes in the PBS group were of donor origin, whereas the engraftment level reached 73.5% and 75.0% ($P \leq .03$), respectively, in the 802-2 and anti-CD4 mAb groups (Figure 3). These results suggested that a single injection of 802-2 peptide analogue at the time of transplantation resulted in significant enhancement of donor engraftment in the lymphoid compartment.

Effect of 802-2 Peptide on Donor T-Cell Reconstitution

To asses the effect of the 802-2 peptide on the degree of donor $CD4^+$ and $CD8^+$ T-cell subset reconstitution in bm12 ATBM-transplanted B6-CD45.1 recipients after PBS, 802-2, or anti-CD4 mAb treatment, splenocytes were initially analyzed by flow cytometry on day 30 after BMT. At this time point, there was a significant increase in the $CD4^+$ T-cell donor compartment after 802-2 peptide treatment compared with the PBS-treated group (from 36% to 56%, respectively; $P < .002$; Figure 4A). A similarly significant increase was observed in mice that received anti-CD4 mAb treatment (58%). Among the $CD8^+$ T cells, there

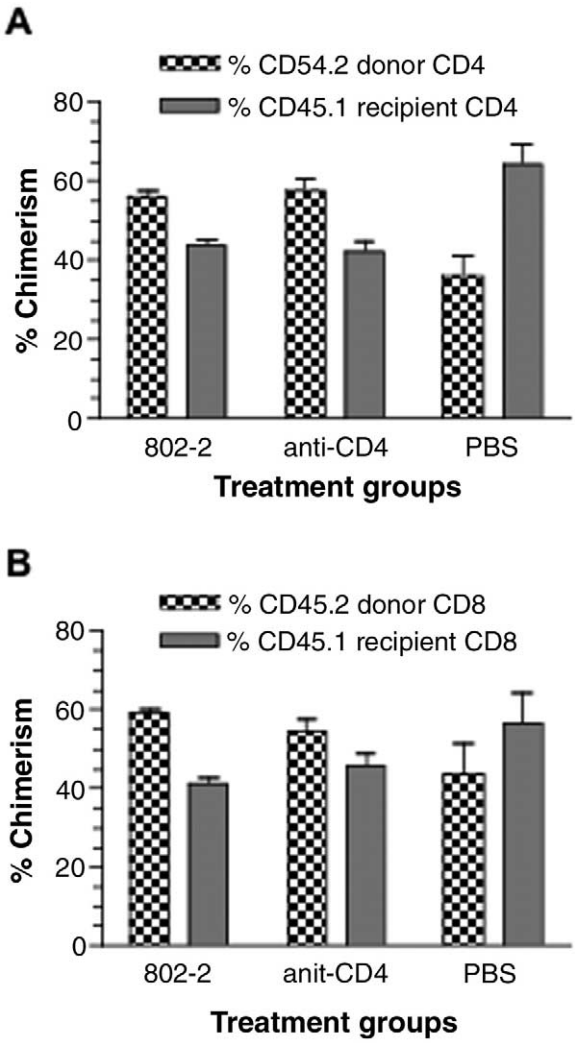

Figure 4. Donor CD4$^+$ and CD8$^+$ T-cell reconstitution in spleens 30 days after BMT. B6-CD45.1 mice underwent transplantation with bm12 ATBM and were treated as described in Figure 2. CD4$^+$ and CD8$^+$ T cells from recipient spleens (n = 3-5) were analyzed for donor chimerism and T-cell phenotype on day 30 after BMT. A, The donor expressions in the 802-2 peptide (56%) and anti-CD4 mAb (57.8%) treatment groups were significantly different from that of the PBS control group (36.0%; $P < .002$). B, The donor CD8$^+$ T-cell values in the 802-2 peptide (59%) and anti-CD4 mAb (54.4%) treatment groups were marginally significantly different from that of the PBS control group (43.4%; $P \leq .09$). The data (mean ± SD) are from 1 representative experiment of 2 performed.

was a moderate, but only marginally significant ($P \leq .09$), increase in the donor compartment in the 802-2 (59.0%) and anti-CD4 (54.4%) groups compared with that of the PBS control (43.4%; Figure 4B).

On day 72 after BMT, the increases in the levels of the donor-derived CD4$^+$ and CD8$^+$ T-cell populations were still apparent in the spleens of the respective 802-2–treated (61% and 64%) and anti-CD4 mAb–treated (65% and 66%) groups compared with the PBS group (50% and 58%; data not shown). This later time point also afforded an adequate opportunity to examine lymph node reconstitution, and it was found that the donor-derived CD4$^+$ T cells increased significantly ($P \leq .039$) in the 802-2–treated and anti-CD4 mAb–treated recipients (81% and 76%, respectively) compared with the PBS-treated group (61%; Figure 5A). Donor CD8$^+$ T-cell chimerism was also significantly increased ($P \leq .002$) from 57.6% in the PBS group to 81.6% and 74.4% in the 802-2–treated and anti-CD4–treated groups, respectively (Figure 5B).

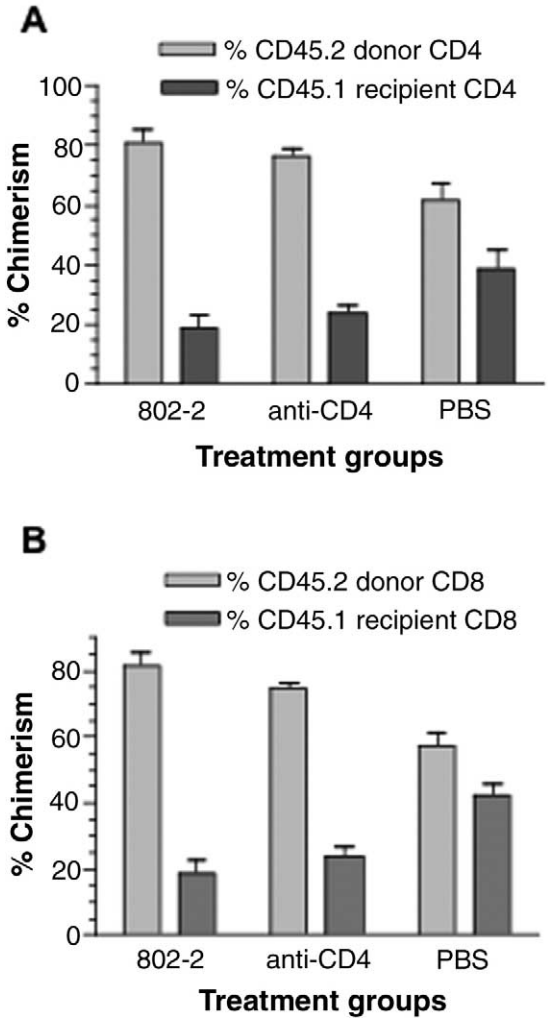

Figure 5. Donor CD4$^+$ and CD8$^+$ T-cell reconstitution in lymph nodes 72 days after BMT. B6-CD45.1 mice underwent transplantation with bm12 ATBM and were treated as described in Figure 2. CD4$^+$ and CD8$^+$ T cells from recipient (n = 3-5) lymph nodes were analyzed for donor chimerism on day 72 after BMT by flow cytometry. A, The donor-derived CD4$^+$ T-cell values in the 802-2 peptide (81%) and anti-CD4 mAb treatment (76%) groups were significantly higher than in the PBS control group (61%; $P \leq .04$). B, Donor CD8$^+$ T-cell chimerism was also significantly increased ($P \leq .002$), from 57.6% in the PBS group to 81.6% and 74.4% in the 802-2–treated and anti-CD4–treated groups, respectively. The data (mean ± SD) are from 1 representative experiment of 2 performed.

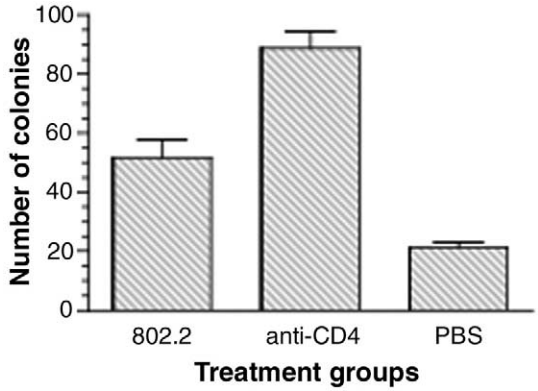

Figure 6. Effect of 802-2 peptide on CFU-GM colony formation. B6-CD45.1 mice underwent transplantation with bm12 ATBM and were treated as described in Figure 2. On day 5 after BMT, splenocytes were pooled from 3 B6-CD45.1 mice per treatment group, plated in triplicate in semisolid methylcellulose media, and cultured for 6 days. Colonies that contained >30 CFU-GM cells were scored as positive. Increased numbers of colonies were formed in splenocyte cultures from the 802-2 peptide–treated (2.4-fold) and the anti-CD4 mAb–treated (4.1-fold) groups in comparison to the PBS control group ($P \leq .001$). The results are from 1 representative experiment of 3 performed.

Hematopoietic Progenitor Cell Engraftment in 802-2 Peptide–Treated Mice

The potential to form granulocyte-macrophage colonies (CFU-GM) within the first week after donor ATBM transplantation is a major sign of successful engraftment [17,18]. Thus, the effect of 802-2 peptide administration on the enhancement of bm12 donor–derived CFU-GM was investigated. Splenocytes were pooled from 3 B6-CD45.1 mice per treatment group (0.2 mL of PBS IV, 0.5 mg of 802-2 in 0.2 mL IV, and 25 μg of anti-CD4 mAb in 0.2 mL IP) on day 5 after BMT and cultured for 6 days in semisolid methylcellulose medium to develop the CFU-GM colonies. Increased numbers of CFU-GM colonies were formed in splenocyte cultures from the 802-2 peptide–treated (2.4-fold) and the anti-CD4 mAb–treated (4.1-fold) groups in comparison to the PBS control group ($P \leq .001$; Figure 6). The CFU-GM progenitor cells were further analyzed for donor/host origin by 2-color flow cytometry with FITC–anti-CD45.1 and PE–anti-CD45.2 mAb. Colonies were found to be >90% donor origin in all of the treatment groups (data not shown). Overall, the results of the CFU-GM assay supported the broad augmentation of donor hematopoietic cell engraftment at the early post-BMT stages after a single injection of the 802-2 peptide.

Effect of 802-2 Peptide on Donor Marrow Engraftment in Presensitized Recipients

In human BMT, prior blood transfusions and parity may presensitize the patients to donor histocompatibility antigens and thereby increase the risk of graft rejection [19]. To assess the capacity of the 802-2 peptide to inhibit host resistance to engraftment in this more aggressive type of situation, B6-CD45.1 mice were presensitized with 2×10^7 bm12 splenocytes by IP immunization 14 days before transplantation. On the day of transplantation, the recipient B6-CD45.1 mice were irradiated (800 cGy) and injected with 10^7 bm12 ATBM cells. Preliminary experiments with a single treatment of 802-2 peptide or anti-CD4 mAb on the day of transplantation (previously found effective in naive recipients, as in Figure 2) resulted in only minimal enhancement of donor chimerism in the spleen, as measured on day 30 (data not shown). In an attempt to maximize the effect of the peptide, in subsequent presensitization experiments, recipient mice were given 802-2 (0.5 mg IV) on each of days 0 to 3 after BMT. This treatment schedule caused a significant increase in donor chimerism compared with the PBS-treated control mice (62% versus 15%; $P \leq .007$; Figure 7). It is interesting to note that only weak and insignificant ($P > .05$) enhancement of engraftment was observed in mice treated with multiple doses of anti-CD4 mAb (days 0-3; 29% donor chimerism). Thus, in the case of presensitized recipient mice, treatment with the 802-2 peptide proved to be solely effective in overriding the host memory $CD4^+$ T-cell response and enhancing donor engraftment.

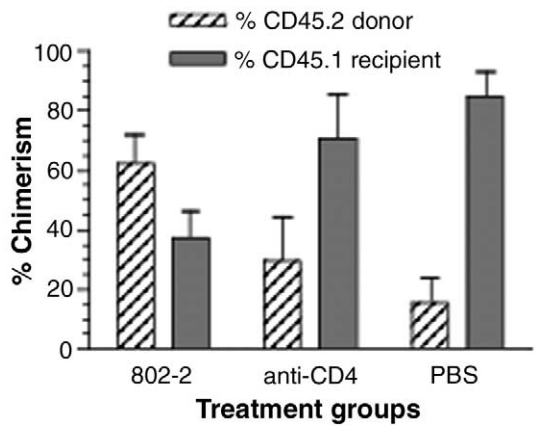

Figure 7. Effect of 802-2 peptide on donor chimerism in presensitized recipients. B6-CD45.1 mice were presensitized (IP) with 2×10^7 bm12 splenocytes 14 days before irradiation exposure (800 cGy) and transplantation with bm12 ATBM (10^7 cells). Recipients were treated daily from day 0 to 3 with PBS, 802-2 peptide (0.5 mg), or anti-CD4 mAb. Donor chimerism was analyzed 30 days after BMT by flow cytometry with FITC–anti-CD45.1 and PE–anti-CD45.2 mAb. This treatment schedule caused a significant increase in donor chimerism in the 802-2–treated group (62%) compared with the PBS-treated control mice (15%; $P \leq .007$). The data (mean ± SD) are from 1 representative experiment (n = 5 mice per group) of 2 performed.

DISCUSSION

In this article, we have described a novel approach that uses a small cyclic heptapeptide, which is able to disrupt the function of the CD4 molecule during the activation process and specifically inhibit activated alloreactive $CD4^+$ T cells. This approach provides a more targeted therapy as part of a posttransplantation immunomodulating treatment regimen to promote hematopoietic stem cell engraftment. The 802-2 peptide was able to significantly enhance donor marrow engraftment across an MHC class II barrier in sublethally irradiated recipients, in which residual host $CD4^+$ T cells can mediate graft rejection [16]. The levels of donor chimerism achieved after a single injection of 802-2 peptide treatment at the time of transplantation in nonsensitized recipients were nearly equivalent to those reached with pan-depleting anti-CD4 mAb treatment.

The inhibition of $CD4^+$ T-cell responses by treatment of recipient mice with mAb directed against the CD4 molecule effectively decreases the incidence of GVHD and graft rejection after transplantation [20]. However, mAb treatment for other diseases has been associated with several problems in the clinical setting. Long-term treatment of autoimmune patients with varying anti-CD4 mAbs has resulted in general immunosuppression and an increased risk of opportunistic infections [21,22]. Shorter-term treatments with mAb may be more promising from a safety point of view because they allow the CD4 compartment to recover more quickly [23]. In addition, some laboratories have reported the inability of anti-CD4 mAb treatments to inhibit memory T-cell responses [24-27]. Murine antibodies have also been found to induce human anti-mouse immunoglobulin responses that can limit the effectiveness and prolonged application of these treatments [28].

In contrast, the 802-2 peptide confers several advantages over anti-CD4 mAb. The peptide does not seem to be immunogenic and has failed to generate antibody responses upon repeated injection in mice (unpublished data). Most importantly, there was no significant diminution in the size of the $CD4^+$ T-cell subset within days after 802-2 administration, and the $CD4^+$ T cells were fully functional, as evidenced by their capacity to respond to both recall antigens and third-party alloantigens [12]. Perhaps the most significant finding, with relevance to the human BMT situation, was that 802-2, in contrast to mAb, was also capable of inhibiting the anti-donor activity of presensitized $CD4^+$ host T cells (Figure 7). The half-life retention of 802-2 in the serum of mice is approximately 12 minutes, although it is unclear how long the peptide may be concentrated in the peripheral lymphoid system (unpublished data, 2004). This potentially short window of efficacy might account for the observation that multiple treatments over the first 3 days after transplantation were much more effective than a single dose in the inhibition of presensitized host T-cell rejection of the marrow graft (Figure 7).

Pretransplantation immunization of recipients via blood transfusion may increase the incidence of marrow graft rejection, and this issue has always been a primary concern for patients with aplastic anemia [29,30]. In leukemia patients receiving allogeneic BMT, the development of graft failure varies depending on several factors, including the type of preconditioning and immunosuppression, the strength of the HLA and non-HLA histocompatibility antigen disparities involved, presensitization of the recipients, and sex mismatch in female recipients [31-33]. In addition, the advent of approaches involving T-cell depletion of bone marrow to prevent GVHD significantly increased the incidence of graft failure with HLA-identical transplant situations [33,34] and with matched unrelated marrow donors [35], theoretically because of the lack of donor alloreactive responses to retard the residual host immune elements responsible for rejection. In recent years, the development of nonmyeloablative approaches with reduced overall transplant-related toxicity to the recipients has gained much interest, but it also increases the risk of graft failure [4-6]. Because 1 of the main aims of these reduced-intensity conditioning regimens is to reach a state of clinical tolerance by allowing establishment of a mixed chimeric immune system [36], the targeting of alloreactive T cells from either the donor or host compartment would seem to be an essential component. Theoretically, the use of the 802-2 peptide analogue, together with a nonmyeloablative approach, could optimize the induction of tolerance through inhibition of all alloreactive $CD4^+$ T cells and markedly reduce the incidence of morbidity and mortality in the early posttransplantation period.

In the clinical setting for HLA-identical transplantations, marrow rejection seems to be mediated primarily by $CD8^+$ T cells [37]. In HLA-mismatched situations, both humoral and cellular mechanisms may cause rejection, the latter predominantly because of $CD4^+$ and $CD8^+$ T cells directed to MHC class II and class I antigens, respectively [38,39]. The distinctive role of T-cell subsets in graft failure has also been clearly demonstrated experimentally in MHC-mismatched strain combinations, including the bm12 → B6-CD45.1 model used in this study [16]. In light of the potential importance of host presensitization to graft rejection mediated by both T-cell subsets, it is significant that the 802-2 peptide analogue was able to substantially block the response of the primed host to MHC class II–disparate donor marrow (Figure 7).

Related investigations with the 802-2 peptide in murine models for experimental allergic encephalomyelitis, skin graft rejection, and GVHD have dem-

onstrated inhibitory effects on the development of $CD4^+$ T cell–mediated disease [11,12]. Furthermore, in the experimental allergic encephalomyelitis study, it was found that 802-2 could affect secondary T-cell responses, thus suggesting that even memory T cells could be disrupted in their activation process by the peptide [12]. On the basis of computer modeling of the CD4/MHC class II interactions, it has been proposed that the CDR3-CC′ loop region on the first domain of the CD4 molecule forms a binding pocket that allows for oligomerization of CD4/MHC class II complexes; this, in turn, enhances the cross-linking of T-cell receptors necessary for T-cell activation [10]. The 802-2 peptide is a structural mimic of the CC′ loop and is thus hypothesized to interfere with stable oligomerization. Recent preliminary experiments support this notion in that the presence of 802-2 during exposure of $CD4^+$ T-cell receptor transgenic cells to a B-cell line presenting appropriate antigen results in a significant decrease of synapse formation and $p56^{lck}$ tyrosine kinase activity, whereas fyn activity seems to be undiminished (Sarma and Korngold, unpublished data, 2004). In addition, this disturbance in the activation cascade is thought to then lead either to a form of anergy or to apoptosis of the responding T cell, the latter indicated by the observation of a significant increase of poly(adenosine diphosphate ribose) polymerase (PARP) cleavage and caspase 3 activity 3 days after exposure to antigen and 802-2.

It is not yet understood whether the mechanism of action of 802-2 is the same for memory T cells as it is for naive T cells in the presence of antigen. However, it is clear from the presensitized recipient experiments (Figure 7) that 802-2 treatment in vivo is more effective than anti-CD4 mAb at inhibition of memory T cells, and this would suggest that the mechanisms of action are indeed different between the 2 agents. Chace et al. [25] investigated the inability of anti-CD4 mAb to delete interleukin $2R^+$ CD4 T cells in vivo and suggested that the antibody mediates a negative signal through the CD4-$p56^{lck}$ molecule which can be overridden by T-cell receptor engagement. However, as stated previously, 802-2 may operate by reducing synapse formation and causing an imbalance between $p56^{lck}$ and fyn signaling; the imbalance leads to disruption of the T-cell activation process and subsequent anergy or apoptosis. This mechanism may thus be operative for both naive and memory T cells.

In this study, we hypothesize that the 802-2 peptide largely inhibits the residual host alloreactive $CD4^+$ T cells that are being activated (or reactivated, in the case of presensitized cells) early after transplantation, thus rendering them incapable of mounting an attack against donor hematopoietic stem cells and their differentiated progeny. With the short serum half-life of the 802-2 peptide in vivo, it is reasonable to suggest the possibility that, if administered only within the first week of transplantation, the peptide could specifically inhibit both host-versus-graft–reactive and GVHD-reactive $CD4^+$ T cells while leaving the remaining nonalloreactive $CD4^+$ T-cell populations intact for subsequent development of responses to opportunistic infections or potential leukemic relapse.

In summary, the 802-2 peptide seems to be an effective agent for the prevention of marrow graft rejection as a result of MHC class II incompatibilities after a reduced-intensity conditioning regimen and, as such, may serve as a platform for the development of phase I studies to test its efficacy in the clinical BMT setting.

ACKNOWLEDGMENTS

We are grateful for the expert technical assistance of Tim Mulligan and Christine DiRienzo. This work was supported by US Public Health Service Research grant no. P01 CA77401 from the National Cancer Institute, National Institutes of Health.

REFERENCES

1. Blazar B, Korngold R, Vallera DA. Recent advances in graft-versus-host disease prevention. *Immunol Rev*. 1997;157:79-109.
2. Reddy P, Ferrara JL. Immunobiology of acute graft-versus-host disease. *Blood Rev*. 2003;17:187-194.
3. Butturini A, Gale RP. Clinical trials of T-cell depletion: current controversies, future directions. In: Gale RP, Champlin RA, eds. *Bone Marrow Transplantation: Current Controversies* (UCLA Symposia on Molecular and Cellular Biology, Vol 91). New York: Alan R. Liss; 1989:495-509.
4. McSweeney PA, Niederwieser D, Shizuru JA, et al. Haematopoietic cell transplantation in older patients with hematologic malignancies: replacing high-dose cytotoxic therapy with graft-versus-tumor effects. *Blood*. 2001;97:3390-4000.
5. Bornhauser M, Thiede C, Platzbecker U, et al. Dose-reduced conditioning and allogeneic hematopoietic stem cell transplantation from unrelated donors in 42 patients. *Clin Cancer Res*. 2001;7:2254-2262.
6. Maris MB, Niederwieser D, Sandmaier BM, et al. HLA-matched unrelated donor hematopoietic cell transplantation after nonmyeloablative conditioning for patients with hematologic malignancies. *Blood*. 2003;102:2021-2030.
7. Schwartz RS. The new immunology—the end of immunosuppressive drug therapy? *N Engl J Med*. 1999;340:1754-1756.
8. Larsen CP, Elwood ET, Alexander DZ, et al. Long term acceptance of skin and cardiac allografts after blocking CD40 and CD28 pathways. *Nature*. 1996;381:434-438.
9. Li Y, Li XC, Zheng XX, Wells AD, Turka LA, Storm TB. Blocking both signal 1 and signal 2 of T-cell activation prevents apoptosis of alloreactive T cells and induction of peripheral allograft tolerance. *Nat Med*. 1999;5:1298-1302.
10. Li S, Satoh T, Korngold R, Huang Z. CD4 dimerization and oligomerization: implications for T-cell function and structure-based drug design. *Immunol Today*. 1998;19:455-462.
11. Satoh T, Aramini JM, Li S, et al. Bioactive peptide design based

on protein surface epitopes. A cyclic heptapeptide mimics CD4 domain 1 CC′ loop and inhibits CD4 biological function. *J Biol Chem*. 1997;272:12175-12180.
12. Edling AE, Choksi S, Huang Z, Korngold R. Effect of a synthetic peptide mimic of the human CD4 domain 1 CC′-loop region on murine experimental allergic encephalomyelitis: inhibition of both primary and secondary responses. *J Neuroimmunol*. 2001;112:115-128.
13. Bruce J, Symington FW, McKearn TJ, Sprent J. A monoclonal antibody discriminating between subsets of T and B cells. *J Immunol*. 1981;127:2496-2501.
14. Dialynas DP, Wilde DB, Marrack P, et al. Characterization of the murine antigenic determinant, designated L3T4a, recognized by monoclonal antibody GK1.5: expression of L3T4a by functional T cell clones appears to correlate primarily with class II MHC antigen-reactivity. *Immunol Rev*. 1983;74:29-56.
15. Unkeless JC. Characterization of a monoclonal antibody directed against mouse macrophage and lymphocyte Fc receptors. *J Exp Med*. 1979;150:580-596.
16. Vallera DA, Taylor PA, Sprent J, Blazar BR. The role of host T cell subsets in bone marrow rejection directed to isolated major histocompatibility complex class I versus class II differences of bm1 and bm12 mutant mice. *Transplantation*. 1994;57: 249-256.
17. Fujimori Y, Hara H, Nagai K. Effect of lymphokine-activated killer cell fraction on the development of human hematopoietic progenitor cells. *Cancer Res*. 1987;48:534-538.
18. Spitzer G, Verma DS, Fisher R, et al. The myeloid progenitor cell—its value in predicting hematopoietic recovery after autologous bone marrow transplantation. *Blood*. 1980;55:317-323.
19. Storb R, Weiden PL. Transfusion problems associated with transplantation. *Semin Hematol*. 1981;18:163-176.
20. Cobbold S, Martin G, Waldmann H. Monoclonal antibodies for prevention of graft-versus-host disease and marrow graft rejection. The depletion of T cells subsets in vitro and in vivo. *Transplantation*. 1986;42:239-247.
21. Racadot E, Rumbach L, Bataillard M, et al. Treatment of multiple sclerosis with anti-CD4 monoclonal antibody: a preliminary report on B-F5 in 21 patients. *J Autoimmun*. 1993;6: 771-786.
22. Lindsey JW, Hodgkinson S, Mehta R, Mitchell D, Enzmann D, Steinman L. Repeated treatment with chimeric anti-CD4 antibody in multiple sclerosis. *Ann Neurol*. 1994;36:183-189.
23. Choy EH, Connolly DJ, Rapson N, et al. Pharmacokinetic, pharmacodynamic and clinical effects of a humanized IgG1 anti-CD4 monoclonal antibody in the peripheral blood and synovial fluid of rheumatoid arthritis patients. *Rheumatology*. 2000;39:1139-1146.
24. Weyand CM, Goronzy J, Swarztrauber K, Fathman CG. Immunosuppression by anti-CD4 treatment in vivo. Persistence of secondary antiviral immune responses. *Transplantation*. 1989; 47:1039-1042.
25. Chace JH, Cowdery JS, Field EH. Effect of anti-CD4 on CD4 subsets. I. Anti-CD4 preferentially deletes resting, naive CD4 cells and spares activated CD4 cells. *J Immunol*. 1994;152:405-412.
26. Rep MH, van Oosten BW, Roos MT, Ader HJ, Polman CH, van Lier RA. Treatment with depleting CD4 monoclonal antibody results in a preferential loss of circulating naive T cells but does not affect IFN-gamma secreting TH1 cells in humans. *J Clin Invest*. 1997;99:2225-2231.
27. Llewellyn-Smith N, Lai M, Miller DH, Rudge P, Thompson AJ, Cuzner ML. Effects of anti-CD4 antibody treatment on lymphocyte subsets and stimulated tumor necrosis factor alpha production: a study of 29 multiple sclerosis patients entered into a clinical trial of cM-T412. *Neurology*. 1997;48:810-816.
28. Hafler DA, Ritz J, Schlossman SF, Weiner HL. Anti-CD4 and anti-CD2 monoclonal antibody infusions in subjects with multiple sclerosis. Immunosuppressive effects and human anti-mouse responses. *J Immunol*. 1988;141:131-138.
29. Champlin RE, Horowitz MM, van Bekkum DW, et al. Graft failure following bone marrow transplantation for severe aplastic anemia: risk factors and treatment results. *Blood*. 1989;73: 606-613.
30. Storb R, Blume KG, O'Donnell MR, et al. Cyclophosphamide and antithymocyte globulin to condition patients with aplastic anemia for allogeneic marrow transplantations: the experience in four centers. *Biol Blood Marrow Transplant*. 2001;7:39-44.
31. Hows JM. Mechanisms of graft failure after human marrow transplantation: a review. *Immunol Lett*. 1991;29:77-80.
32. Vogt MH, Goulmy E, Kloosterboer FM, et al. UTY gene codes for an HLA-B60-restricted human male-specific minor histocompatibility antigen involved in stem cell graft rejection: characterization of the critical polymorphic amino acid residues for T-cell recognition. *Blood*. 2000;96:3126-3132.
33. Kernan NA, Bordignon C, Heller G, et al. Graft failure after T-cell-depleted human leukocyte antigen identical marrow transplants for leukemia: I. Analysis of risk factors and results of secondary transplants. *Blood*. 1989;74:2227-2236.
34. O'Reilly RJ, Collins NH, Kernan N, et al. Transplantation of marrow-depleted T cells by soybean lectin agglutination and E-rosette depletion: major histocompatibility complex-related graft resistance in leukemic transplant recipients. *Transplant Proc*. 1985;17:455-462.
35. Beatty PG, Clift RA, Mickelson EM, et al. Marrow transplantation from related donors other than HLA-identical siblings. *N Engl J Med*. 1985;313:765-771.
36. Salamal AD, Remuzzi G, Harmon WE, Sayegh MH. Challenges to achieving clinical transplantation tolerance. *J Clin Invest*. 2001;108:943-948.
37. Bordignon C, Keever CA, Small TN, et al. Graft failure after T-cell-depleted human leukocyte antigen identical marrow transplants for leukemia: II. In vitro analyses of host effector mechanisms. *Blood*. 1989;74:2237-2243.
38. Barge AJ, Johnson G, Witherspoon R, Torok-Storb B. Antibody-mediated marrow failure after allogeneic bone marrow transplantation. *Blood*. 1989;74:1477-1480.
39. Donohue J, Homge M, Kernan NA. Characterization of cells emerging at the time of graft failure after bone marrow transplantation from an unrelated marrow donor. *Blood*. 1993;82: 1023-1029.